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REGULATION OF AUTOPHAGY AND MTOR DURING SEMLIKI FOREST VIRUS INFECTION

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About the cover:
The cytoplasm-to-vacuole targeting (Cvt) pathway in yeast directs membrane from a mitochondrion (top right) to a forming Cvt vesicle (center) before the Cvt vesicle fuses with the vacuole (bottom right). The Cvt pathway shares many similarities with autophagy.
Original painting by David S. Goodsell with scientific input from Daniel J. Klionsky. Previously published as cover art accompanying He et al. (2006) J. Cell Biol. 175:925-935. Reproduced with kind permission from David S. Goodsell.

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ABSTRACT

Semliki Forest virus (SFV) infection causes dramatic changes to the infected cell. For example, synthesis of most cellular proteins shuts off within 4 hours. The cell launches an antiviral response, forms stress granules and, as we found in the work leading up to this thesis, accumulates autophagosomes. Studies on various host responses to viral infections are relevant for understanding both viral pathogenesis and innate immunity against viruses.

In the past decade autophagy has gained prominence as a protein degradation system, and the autophagy field is only just beginning to understand how this cellular mechanism is modulated during viral infection, and what role it plays either in host cell defence, or in supporting viral replication. To be better equipped for studying autophagy, we developed a flow cytometry-based method for quantifying autophagosomes, in **Paper I**. The method was quicker and less subjective than most pre-existing methods. Then, in **Paper II** we investigated autophagy during SFV infection, and found that autophagosomes accumulated during infection not due to increased synthesis of new autophagosomes, but due to a decreased rate of degradation of autophagosomes. We also found that expression of the SFV glycoproteins was necessary for the accumulation of autophagosomes.

Our work on autophagy led us to investigate the status of mTOR, a regulator of autophagy, during SFV infection. mTOR is normally active under nutrient rich conditions, and inactive under nutrient starvation conditions. In **Paper III**, we showed that SFV infection caused mTOR to remain active during nutrient starvation. Unlike the effect on autophagosome accumulation, this effect on mTOR did not depend on expression of the SFV glycoproteins. Despite the maintenance of mTOR activity in SFV infected cells, inhibition of mTOR activity with rapamycin had no effect on SFV growth rate.

Collectively, the results presented in this thesis provide a novel, practical tool for measuring autophagy, as well as insight on how autophagy and its regulator mTOR are modulated during SFV infection.

LIST OF PUBLICATIONS

I. **Kai Eng**, Marc D. Panas, Gunilla B. Karlsson Hedestam and Gerald M. McInerney. 2010.
A novel quantitative flow cytometry-based assay for autophagy.
Autophagy 6: 634-641

II. **Kai Eng**, Marc D. Panas, Deirdre Murphy, Gunilla B. Karlsson Hedestam and Gerald M. McInerney. 2012.
Accumulation of autophagosomes in Semliki Forest virus-infected cells is dependent on expression of the viral glycoproteins.
Journal of Virology 86: 5674-5685

III. **Kai Eng***, Roberta Biasiotto* and Gerald M. McInerney. (*Joint first authors.) 2012.
Semliki Forest virus infection maintains mTOR activity during nutrient starvation.
Manuscript

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LIST OF ABBREVIATIONS

4E-BP1	eukaryotic translation initiation factor 4E (eIF4E) binding protein 1
AMPK	AMP-activated kinase
Atg	autophagy-related
EBSS	Earle's balanced salt solution
EGFP	enhanced green fluorescent protein
eIF2α/eIF4E/ eIF4F/eIF4G	eukaryotic translation initiation factor 2α/4E/4F/4G
ER	endoplasmic reticulum
FIP200	focal adhesion kinase family-interacting protein of 200 kDa
FKBP12/ FKBP38	FK506-binding protein of 12/38 kDa
G3BP1	GTPase activating protein (SH3 domain) binding protein 1
HCMV	human cytomegalovirus
HCV	hepatitis C virus
HOS	human osteosarcoma
hpi	hours post infection
HSV-1	herpes simplex virus type 1
IFN	interferon
IRF3/IRF7	interferon regulatory factor 3/7
LC3	microtubule-associated protein light chain 3
M2	matrix protein 2
MEF	mouse embryonic fibroblast
MHC	major histocompatibility complex
Mnk1	mitogen-activated protein (MAP) kinase signal-integrating kinase 1
mRFP	monomeric red fluorescent protein
(m)TOR	(mammalian) target of rapamycin
nsP	non-structural protein
PAR	poly(ADP-ribose)
PDK1	phosphoinositide-dependent protein kinase 1
PE	phosphotidylethanolamine
PI3K	phosphatidylinositol 3-kinase
PKR	protein kinase R

Rag	Ras-related GTP-binding
Rheb	Ras homolog enriched in brain
RIG-I	retinoic-acid inducible gene I
Rubicon	RUN domain Beclin-1 interacting and cystein-rich containing protein
S6	ribosomal protein S6
S6K	p70 S6 kinase
SFV	Semliki Forest virus
SQSTM1	sequestosome 1
TIA-1	T-cell-restricted intracellular antigen-1
TLR7	Toll-like receptor 7
TOS	target of rapamycin (TOR) signalling
TSC	tuberous sclerosis complex
ULK1	unc-51 like kinase 1
UVRAG	UV radiation resistance associated gene
Vps34	vacuolar protein sorting-associated protein 34
VSV	vesicular stomatitis virus
Xbp-1	X-box-binding protein 1

1 INTRODUCTION

1.1 SEMLIKI FOREST VIRUS

1.1.1 Background

Semliki Forest virus (SFV) was first isolated in the Semliki Forest of Uganda in 1942 (131). It is an insect-borne virus, spread by mosquitoes and infecting small animals. The virus is neurotropic in these animals. SFV is not an important human pathogen. However, it is closely related to the emerging Chikungunya virus, which has recently caused outbreaks of severe illness in human populations.

Both SFV and Chikungunya virus belong to the *Alphavirus* genus, family *Togaviridae*. A third *Alphavirus*, Sindbis virus, is also closely related to SFV. SFV and Sindbis virus have both been used extensively in laboratories as model viruses for studying the biology of viral infection, not least due to their broad host range and efficient replication in laboratory cell lines. In this thesis I will discuss what we have observed in SFV infected cells, in comparison with the published literature on Chikungunya virus and Sindbis virus infected cells.

SFV is also well known in the biotechnology field as a viral vector for delivery of foreign genes for expression of heterologous proteins, potentially useful for vaccination or gene therapy. Systems to generate suicidal virus particles have been developed: first, a gene of interest is inserted, in place of the genes encoding SFV structural proteins, into a plasmid encoding a cDNA copy of the SFV genome, the SFV vector. (For more on the SFV genome, see next section.) Then, RNA from the recombinant SFV vector is synthesised in vitro and co-transfected into cells along with helper RNA encoding the SFV structural proteins. In the co-transfected cells, the packaging signal present on the SFV vector causes packaging of this RNA into particles. These particles are suicidal (causing a single round of infection only) because they do not contain the RNA that codes for SFV structural proteins (78). In the single-round infected cells, heterologous protein encoded by the vector is expressed at high levels under the control of the *Alphavirus* 26S promoter. Subsequently, an improved system was developed that eliminated the risk of the helper RNA recombining with the vector RNA in co-transfected cells to generate wild type SFV RNA, by splitting the helper RNA into two independent RNA molecules that each coded for different SFV structural proteins (130). While the life cycle of SFV has been well defined, further studies on the biology of SFV infected cells may provide insights for future SFV-based technologies.

1.1.2 Structure

SFV has a positive-sense RNA genome of 11.5 kb (Figure 1). This 42S RNA genome is capped on the 5’ end and polyadenylated on the 3’ end. The genome is packed in an

icosahedral capsid. Surrounding the capsid is the SFV envelope, which is derived from the host cell plasma membrane. There are 80 individual glycoprotein spike complexes on the SFV surface, each consisting of three E1-E2 heterodimers, thus there are 240 heterodimers altogether on the viral surface. A third protein, E3, is associated to each dimer. The glycoprotein spikes are anchored in the envelope by the E2 cytoplasmic domain, which interact with the capsid.

The RNA genome has two open reading frames. The 5' two-thirds of the genome code for non-structural proteins (nsPs) 1-4. The 3' one-third, under the control of an internal promoter, is transcribed into a subgenomic 26S mRNA which codes for the structural proteins. The structural proteins traditionally include capsid, E3, E2, the 6 kDa protein 6K, and E1. In 2008, it was discovered that frameshifting occurs within the sequence encoding 6K so that another protein, TransFrame protein, is also expressed (31).

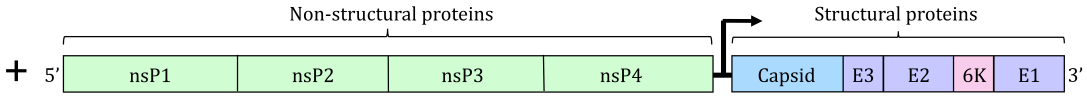

Figure 1

Schematic diagram of the Semliki Forest virus (SFV) genome. The 5' two-thirds of the genome code for non-structural protein 1 (nsP1)-nsP4. The 3' one-third, under the control of an internal promoter (black arrow), codes for the structural proteins.

1.1.3 Viral life cycle

SFV enters the cell via receptor-mediated endocytosis. After SFV is endocytosed, acidification of the endosome causes a change in conformation of the SFV spike, which allows the viral envelope to fuse with the endosomal membrane (144). The E1 spike protein mediates this fusion. After membrane fusion, the nucleocapsid is released into the cytoplasm.

The nucleocapsid then disassembles, and viral RNA is released into the cytoplasm. nsP1234 is translated immediately as a polyprotein. This polyprotein is then sequentially, autocatalytically, cleaved to yield the individual nsP proteins, through the protease activity of nsP2. Viral RNA replication takes place, with the individual nsPs playing various roles (summarised briefly below), at replication complexes assembled on membranous structures.

nsP1 is the capping enzyme for viral genomic and subgenomic mRNA (2). It associates tightly with membranes, and is the membrane anchor for the entire viral replication complex. Immunofluorescence analysis shows that nsP1 is localised to plasma membranes as well as to intracellular membrane-associated replication complexes. Recent work by Spuul et al. showed that nsP1-positive replication complexes are delivered to the plasma membrane before class I phosphatidylinositol 3-kinase (PI3K)-dependent endocytosis back into the cell, revealing a previously unknown large-scale migration of nsP1-positive replication complexes before arrival at intracellular, membrane-associated viral replication sites (132). Tight membrane association of nsP1

can be attributed to its palmitoylated cysteine residues (1), as well as to a segment of amino acids which has high affinity for negatively charged lipids (72), such as phosphatidylserines, which are abundant on the inner leaflet of the plasma membrane.

nsP2 as mentioned earlier is the protease responsible for processing nsP1234 into individual mature proteins. Sequential cleavage by the nsP2 protease regulates the synthesis of negative versus positive strand RNA: nsP123 and nsP4 together promote transcription of negative strand RNA, while subsequent cleavage between nsP1 and nsP23 allows shut-off of negative strand synthesis and a switch to synthesis of positive strand RNA, with preference for genomic RNA. The final cleavage between nsP2 and nsP3 promotes preference for synthesis of the subgenomic mRNA over genomic RNA (65, 76). nsP2 localises both to the nucleus and to the cytoplasm. Its nuclear localisation signal is in its C-terminal domain (114). In a virus expressing a mutant nsP2 which does not translocate to the nucleus, viral spread in adult mouse brain is compromised (30), and synthesis of type I interferon (IFN) by host cells is increased (9).

nsP3 is highly phosphorylated on serines and threonines, by cellular kinases, in its C-terminal domain (107). Little is known about its function in viral replication. Current work from our research group shows that SFV nsP3 binds to GTPase activating protein (SH3 domain) binding protein 1, promoting the disassembly of stress granules, in SFV infected cells (Marc Panas, personal communication and Figure 2). Stress granules are discussed further in section 1.1.4. It has also been reported that the closely related Sindbis virus nsP3 binds to poly(ADP-ribose) (PAR) and PAR polymerase-1 (102), suggesting roles for nsP3 and PAR polymerase-1 in Sindbis virus RNA replication.

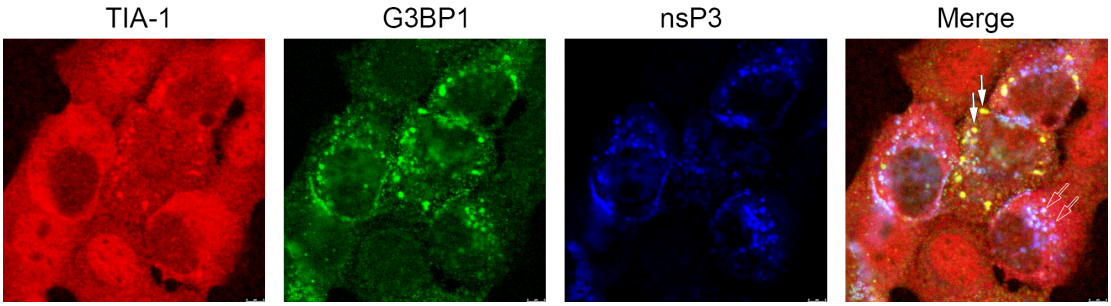

Figure 2

Stress granules in SFV infected cells. Mouse embryonic fibroblasts were infected with SFV and fixed 4 hours later. The stress granule marker T-cell-restricted intracellular antigen-1 (TIA-1) localises to the nucleus in uninfected, nsP3 negative cells, but localises to the cytoplasm in infected, nsP3 positive cells, where it is either associated with GTPase activating protein (SH3 domain) binding protein 1 (G3BP1) in stress granules (yellow puncta, filled arrows) at an early stage of infection, or spread in a diffuse manner in the cytoplasm in later stages of infection. In cells where TIA-1 is diffuse in the cytoplasm, instead of stress granules, nsP3-G3BP1 double-positive foci are observed (purple puncta, open arrows). Microscope images kindly provided by Marc Panas, MTC, Karolinska Institutet.

nsP4 is the RNA-dependent RNA polymerase of SFV; it has segments in its C-terminal domain conserved with all other RNA-dependent RNA polymerases. There are only low levels of nsP4 in an infected cell as it is rapidly degraded in the proteasome after it has matured (85). Although the same has not been shown for SFV, Sindbis virus nsP1234 is synthesised at low levels as a result of a leaky stop codon between nsP123

and nsP4 (133).

After synthesis of viral genomic RNA, a switch to synthesis of subgenomic mRNA occurs as described earlier. The subgenomic mRNA is then translated into a capsid-p62 (precursor of E3 and E2)-6K-E1 polypeptide that gives rise to the SFV structural proteins. Capsid is first autocatalytically cleaved from this polypeptide and binds to the packaging signal of viral genomic RNA, in the cytoplasm (35), while the remaining p62-6K-E1 polypeptide translocates into the endoplasmic reticulum (ER). During translocation, the p62-6K-E1 polypeptide is cleaved by cellular enzymes, forming a p62-E1 dimer. p62 is further cleaved in the Golgi complex into E3 and E2, which remain associated with each other (22). The resulting E1-E2 dimers, with E3 associated, are transported to the plasma membrane.

Capsid interaction with viral genomic RNA in the cytoplasm leads to the formation of nucleocapsids. Nucleocapsids bud out from the plasma membrane, forming progeny virus particles, in a process driven by interactions between the E2 cytoplasmic tail and the capsid protein (57, 135, 151).

1.1.4 Host responses to SFV infection

SFV infection triggers a potent host antiviral response. A prominent virus sensor, and mediator of antiviral response, is protein kinase R (PKR), which recognises intracellular double stranded RNA, assumed to be a replication intermediate in RNA virus infections. Antiviral effects of PKR are reviewed in (38). In the specific case of SFV infection, double stranded RNA is sensed by PKR, which causes a delay in viral protein production and a delay in release of viral progeny, enhancement of the type I IFN response, and promotion of virus clearance, even though it is not PKR that initiates the IFN response (6).

Importantly, activated PKR phosphorylates eukaryotic translation initiation factor 2α (eIF2α), a component of eIF2, inhibiting mRNA translation. This often includes inhibition of translation of viral mRNA. Thus, to dampen IFN production and prevent translational inhibition, as well as to avert other antiviral effects of PKR, many viruses have evolved mechanisms to counter the effects of PKR (reviewed in (73)), such as influenza A virus non-structural protein 1 sequestering any free double stranded RNA (44).

While PKR is a well studied double stranded RNA sensor in SFV infected cells, the retinoic-acid inducible gene I (RIG-I)-like receptor family plays a major role in sensing viral single stranded RNA bearing 5'-phosphates during infection by RNA viruses (110). Members of this family of sensors include RIG-I and melanoma differentiation associated gene 5 (reviewed in (113)). These sensors signal through the adaptor, IFNß promoter stimulator 1 (61), leading to downstream nuclear translocation of the transcription factors interferon regulatory factor 3 (IRF3) and nuclear factor κB, which then leads to production of type I IFN.

IFN was first discovered as a factor which was produced in the supernatant of cells incubated with heat-inactivated influenza virus, and which interfered (thus the name interferon) with the growth of live virus when added to other cells (46). The type I IFNs are composed of several subtypes of IFNα, and IFNß. During type I IFN induction, IFNα4 and IFNß are first produced and secreted. They signal back through the IFNα/ß receptor on the cell surface, causing downstream signalling leading to upregulation of IRF7. When IFN-primed cells are exposed to viruses, they mediate a much greater type I IFN response as IRF7 allows the transcription of all IFNα subtypes and more transcription of IFNß. Downstream signalling cascades, due to engagement of the IFNα/ß receptor, are mediated by Janus kinases and the signal transducer and activator of transcription 1 (Stat1) and Stat2 proteins. The result of type I IFN signalling is transcriptional induction of hundreds of target genes (IFN-stimulated genes), including PKR, which helps cells to achieve an antiviral state (reviewed in (117)).

SFV does not counter the effects of PKR at the level of PKR itself. In an SFV infected cell, PKR is activated and eIF2α is phosphorylated, so translation is generally inhibited, but the subgenomic mRNA of SFV escapes translational shut-off. In Sindbis virus infected cells, this same phenomenon is independent of the presence of subgenomic mRNA (33), suggesting that escape of translation shut-off is not simply caused by out-competition of abundant viral mRNA over host mRNA, and that events earlier than subgenomic mRNA synthesis are responsible for the escape of translational shut-off by viral mRNA. In SFV infected cells, eIF2α phosphorylation leads to the formation of stress granules mediated by the RNA-binding protein T-cell-restricted intracellular antigen-1 ((83) and Figure 2). Stress granules are cytoplasmic complexes of mRNA and protein, where mRNA is stored and sorted while translation is halted, as is the case under stressful conditions such as heat shock. In an SFV infected cell, sequestration of host mRNA but not viral mRNA in stress granules partly contributes to translational shut-off of host but not viral mRNA. Additionally, the viral mRNA has an enhancer sequence which forms a hairpin loop (34), that only works as an enhancer when eIF2α exists mostly in the phosphorylated state (83). Host translational shut-off through inactivation of the eIF2 complex, formation of stress granules that sequester host mRNA, and enhanced translation of SFV mRNA (all dependent on eIF2α phosphorylation), effectively causes an SFV infected cell to become a factory for the synthesis of high levels of SFV structural proteins, while few host proteins are synthesised.

After about 8 hours of productive infection, most of which are assumed to be concurrent with rapid type I IFN production, and the last 4 of which consist of host translational shut-off and high levels of SFV structural protein synthesis, SFV infected cells begin to die via apoptosis. While DNA viruses tend to encode pro-survival proteins, RNA viruses that cause acute infection and quick production of progeny do not need to keep their host cells alive. In the case of SFV infection, RNA replication triggers apoptosis by a Bcl-2 homologous antagonist/killer (Bak)-mediated loss of mitochondrial membrane integrity, cytochrome c release and downstream activation of caspases (142). Further, ER stress due to high levels of synthesis of SFV spike proteins plays a role in accelerating apoptosis via the activation of caspase-12. In cells infected with recombinant SFV lacking genes for the spike proteins, ER stress markers and activated caspase-12 are not present (7).

1.2 AUTOPHAGY

1.2.1 The process of autophagy

Autophagy is a lysosomal (or vacuolar, in yeast) degradation process first described in the budding yeast *Saccharomyces cerevisiae* and later discovered to be largely conserved through all eukaryotic cells. The two main classes of autophagy are chaperone-mediated autophagy, where proteins to be degraded are directly translocated into the lysosome with the help of chaperones (23), and macroautophagy, where proteins or organelles to be degraded are sequestered in double-membrane vesicles termed autophagosomes, which fuse with lysosomes (Figure 3, top). Within lysosomes, proteins are degraded by lysosomal proteases at low pH. In the field of autophagy, the term "autophagy" most often implies macroautophagy, and chaperone-mediated autophagy is referred to by its full name. The type of autophagy this thesis deals with is macroautophagy, hereafter "autophagy" as per convention.

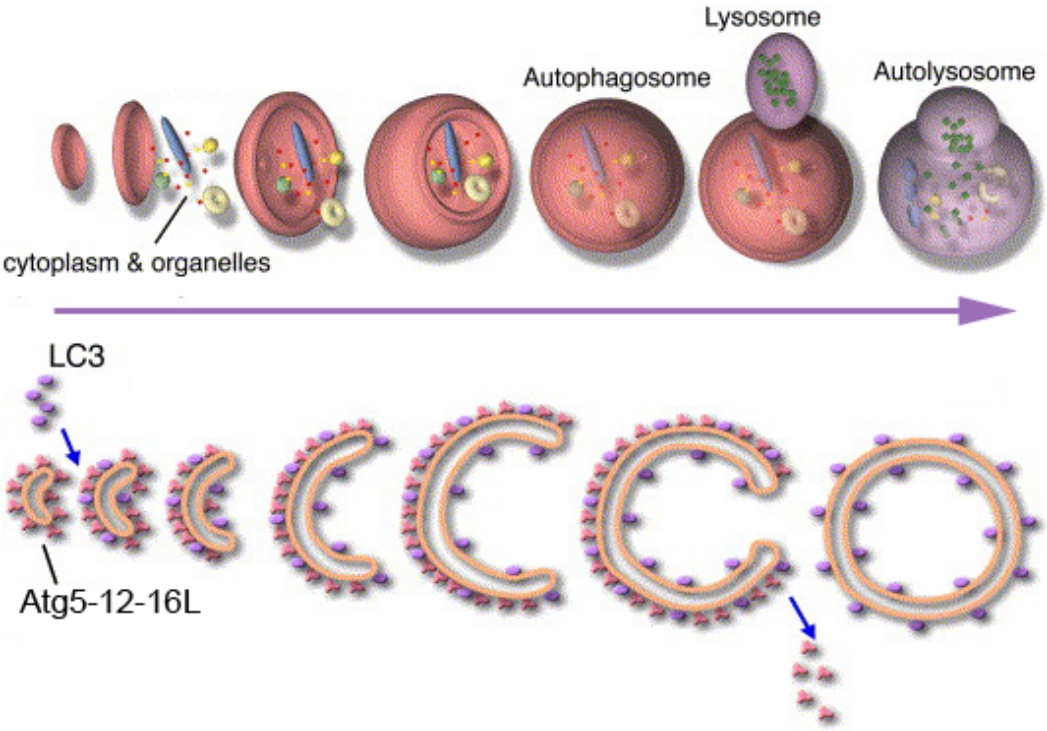

Figure 3
Schematic diagram showing the process of autophagy. Top, a piece of membrane, termed the isolation membrane, is extended until it forms a double-membrane vesicle around cytoplasmic contents: the autophagosome. The autophagosome fuses with a lysosome containing proteases (green circles), forming an autolysosome where autophagic cargo is degraded by proteases. Bottom, autophagy-related 5 (Atg5), Atg12 and Atg16-like (Atg16L) form complexes (pink triangles) that associate with the nascent autophagosome membrane and recruit microtubule-associated protein light chain 3 (LC3) (purple dots). Atg5-12-16L subsequently dissociates from the membrane leaving LC3 associated to both the inner and outer membranes of the autophagosome. Adapted from (150) with permission from Elsevier.

The process of autophagy has been largely delineated in yeast (reviewed in (91)). A large number of autophagy-related (Atg) proteins, many of which are conserved between yeast and mammals, are involved in the autophagic process. The yeast Atg8 protein, or the mammalian homolog microtubule-associated protein light chain 3 (LC3), is associated with the nascent autophagosome membrane and remains associated with the fully formed autophagosome (Figure 3, bottom). Atg8/LC3 is thus the most commonly used marker for autophagosomes.

Notably, Atg12 and Atg8/LC3 are ubiquitin-like proteins that are conjugated to Atg5 and a lipid, phosphotidylethanolamine (PE), respectively. (Unlipidated LC3 and LC3-PE are also known as LC3-I and LC3-II respectively.) The E1-like activation enzyme is Atg7 for both Atg12 and Atg8/LC3, while the E2-like enzyme is Atg10 and Atg3 respectively for Atg12 and Atg8/LC3. After conjugation of Atg12 to Atg5, Atg5 associates with Atg16-like (Atg16L) in mammalian cells. The Atg5-12-16L complex localises to nascent autophagosome membranes and is important for the recruitment of LC3-PE (Figure 3, botom). The deconjugation enzyme Atg4 can reverse Atg8/LC3-PE conjugation, so Atg8/LC3 can be recycled. The ubiquitin-like processes in autophagy are conserved between yeast (90) and mammalian (138) systems.

The main function of autophagy in a normal cell is the constitutive, homeostatic recycling of amino acids through degradation of old or damaged proteins or organelles. Autophagy can be enhanced above these constitutive levels by certain stimuli, such as amino acid starvation. Amino acid starvation upregulates autophagy via the inactivation of yeast target of rapamycin (TOR) or mammalian TOR (mTOR), which represses autophagy under nutrient rich conditions (see section 1.3 for more on mTOR). When mTOR is inactive in starved mammalian cells, unc-51 like kinase 1 (ULK1, homolog of yeast Atg1), as part of the ULK1-Atg13-FIP200 complex, localises to forming autophagosome membranes. Inactivation of mTOR also causes dephosphorylation of ULK1 and Atg13, and promotion of ULK1 kinase activity (36, 56). Both Atg13 and FIP200 (focal adhesion kinase family-interacting protein of 200 kDa) enhance the kinase activity of ULK1 (36). ULK1 kinase activity is required for the initiation of autophagy (reviewed in (87)).

Apart from the ULK1-mediated control of autophagy intiation, autophagy can also be regulated further downstream, via mammalian Beclin 1 (homolog of yeast Atg6). Beclin 1 binds to vacuolar protein sorting-associated protein 34 (Vps34), a class III PI3K involved in intracellular membrane trafficking processes. Beclin 1-Vps34 can further associate with either Atg14 or the UV radiation resistance associated gene (UVRAG) protein in a mutually exclusive manner (48). A subset of Beclin 1-Vps34-UVRAG complex binds to RUN domain Beclin-1 interacting and cystein-rich containing protein (Rubicon). Association of Beclin 1-Vps34 with Atg14 promotes the formation of autophagosome membranes, while association with Rubicon negatively regulates autophagy (153).

Several small molecules are used to regulate autophagy in laboratory experiments. Rapamycin induces autophagy by inactivating mTOR, and small molecules such as wortmannin, LY294002, and 3-methyladenine inhibit autophagy by inhibiting PI3K (8, 126), although the use of PI3K inhibitors has become questionable since they may

inhibit both class III PI3K (which activates autophagy via Beclin 1) and class I PI3K (which inhibits autophagy via downstream activation of mTOR) (109, 147).

Historically autophagy was referred to as a "bulk" protein degradation system, as substrate specificity was not well defined for autophagy compared to the specific, ubiquitin-mediated degradation that occurs via the other major protein degradation system in a cell, proteasomal degradation. Today, this view is challenged as substrates for autophagy are gradually characterised, even though a universal mechanism for targeting substrates to autophagosomes has not emerged. Substrates that have been described for autophagy include poly-ubiquitinated protein aggregates, targeted to autophagosomes via the adaptor p62/sequestosome 1 (SQSTM1) which binds both the aggregates and LC3 (100); cellular organelles, e.g. mitochondria (mitophagy) (4, 16), ribosomes (ribophagy) (69), ER (reticulophagy); and pathogens or components of pathogens (xenophagy) (89). Xenophagy of viruses will be discussed in section 1.2.3.

1.2.2 Methods for monitoring autophagy

A recent article describing assays for monitoring autophagy, and helpful guidelines, can be found at reference (68). I briefly discuss some of these methods to provide the context for the development of our novel method in Paper I.

1.2.2.1 Transmission electron microscopy

Transmission electron microscopy can be used to visualise autophagosomes (Figure 4). This method depends on identification of autophagosomes based on their double-membrane morphology and the presence of cytoplasmic contents within the autophagosome.

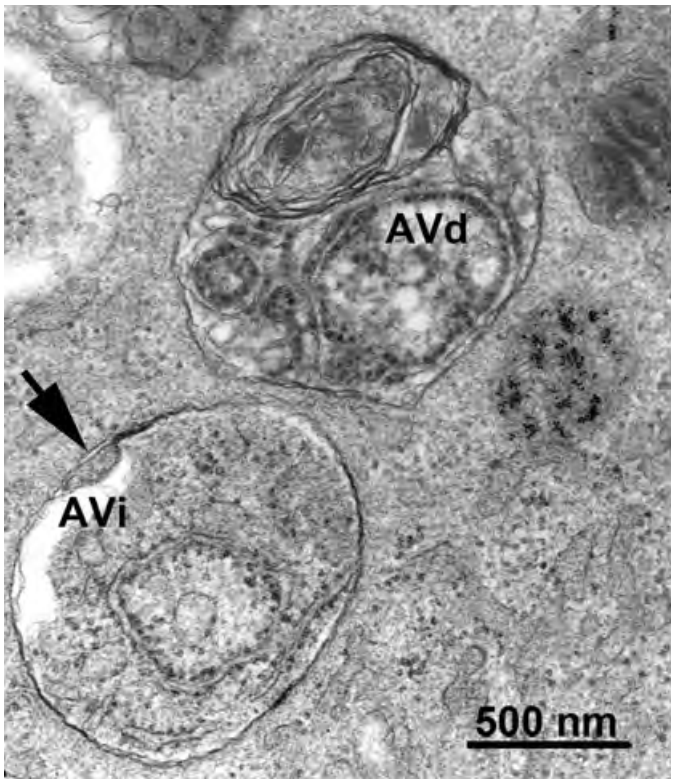

Figure 4
Transmission electron microscopy image of autophagic vacuoles in isolated mouse hepatocytes. The early autophagic vacuole or autophagosome (AVi) is identified by its double-membrane (arrow) and cytoplasmic contents, including ribosomes and rough endoplasmic reticulum (ER). The late, degradative autophagosome (AVd) is identified by its partially degraded contents and rough ER. Adapted from (68) with permission from Landes Bioscience and E.-L. Eskelinen.

1.2.2.2 Western blot for LC3-II band

During the process of autophagy, the autophagosome marker LC3 is converted from the unlipdated LC3-I form to the PE-conjugated LC3-II form (see section 1.2.1). LC3-I runs on an SDS-PAGE gel at an apparent molecular weight of 18 kDa, while LC3-II runs at 16 kDa. The ratio of the intensity of the LC3-II band to that of the LC3-I or actin band is used as a measurement for relative levels of the autophagosome marker LC3-II. In our experience, the LC3-II band is faint in cell lines where the basal amount of autophagosomes is low. The western blot method is not optimal for such cell lines.

1.2.2.3 Fluorescence microscopy

The number of LC3-positive, or enhanced green fluorescent protein (EGFP)-LC3-positive puncta per cell, or percentage of punctate cells within a population of cells, is used to describe the quantity of autophagosomes. This method often depends on manual counting of puncta or cells, and can be both subjective and time-consuming.

1.2.2.4 Flow cytometry-based detection of LC3

A paper by Shvets et al. in 2008 described a method for measuring LC3 levels using flow cytometry (128). This method measured total LC3 levels and did not differentiate between LC3-I and LC3-II. The authors observed that total levels of LC3 declined as a cell was amino acid starved, consistent with the idea that LC3 itself is a substrate for autophagic degradation, thus upregulation of autophagy through starvation decreased the amount of LC3 over time.

In Paper I of this thesis, we developed a method for quantification of autophagosomes that combined the high throughput capacity of flow cytometry, with the advantage of specific detection of LC3-II. See section 3.1 for details.

1.2.2.5 Taking autophagic flux into account

Autophagy is a dynamic process, involving the formation of autophagosomes, transport of autophagosomes to lysosomes, fusion of autophagosomes with lysosomes and degradation of autophagic cargo. As such, quantificaton of a steady-state number of autophagosomes, or a steady-state amount of an autophagosome marker, does not demonstrate the rate of autophagy. For example, from our experience, in a starved human osteosarcoma (HOS) cell compared to a non-starved HOS cell, the rate of autophagosome formation is increased, and the rate of autophagosome degradation increases along with it, resulting in a highly increased rate of autophagy (with a highly

increased rate of amino acid turnover), but little increase in the absolute level of LC3-II.

To circumvent this, it is possible to detect only the formation of new autophagosomes, by blocking the degradation of existing autophagosomes with small molecules that prevent lysosomal acidification. In Paper I, we demonstrated that our novel method for quantifying autophagy was useful for this purpose (see section 3.1).

Other methods that take into account the fact that autophagosomes are constantly formed and degraded have been developed. One such method utilises the tandem reporter monomeric red fluorescent protein (mRFP)-EGFP-LC3 (67) and can tell us if there are changes in the degradation rate of autophagosomes. As EGFP is more sensitive to lysosomal acidic pH than mRFP, the mRFP-EGFP-LC3 reporter fluoresces yellow when it is autophagosome-bound, but fluoresces red when it has been delivered to lysosomes. The ratio of yellow to red puncta thus indicates levels of autophagosomes relative to maturing autolysosomes (autophagosomes fused with lysosomes), and changes in this ratio indicate changes in the fusion rate of autophagosomes with lysosomes. We utilised this method in Paper II to show that fusion of autophagosomes with lysosomes was impaired in SFV infected cells (see section 3.2).

1.2.3 Autophagy and viral infection

Autophagosome accumulation during viral infection has been documented in an increasing number of cases over the past 7 years or so. A non-exhaustive list of viruses that cause this phenomenon, focusing on RNA viruses, include the negative strand RNA viruses measles virus (53) and influenza A virus (37), and the positive strand RNA viruses dengue virus (101), Sindbis virus (96), Chikungunya virus (54, 55, 70), hepatitis C virus (HCV) (129), coronaviruses (20, 111) and poliovirus (51, 140).

Sindbis virus and Chikungunya virus belong to the same genus (*Alphavirus*) as SFV. Due to their relatedness with SFV a brief description of the literature on autophagy and these two viral infections is in order. In Sindbis virus infected cells, the presence of Atg5 protects against lethal infection in the mouse central nervous system. p62/SQSTM1 reportedly interacts with Sindbis virus capsid protein and targets it for degradation in autophagosomes (96). In Chikungunya virus infected cells, levels of ER stress and reactive oxygen species are both increased, and both contribute to increased autophagic flux, with the reactive oxygen species promoting autophagy via inhibition of mTOR (54, 55, 70). When autophagy is inhibited through the use of 3-methyladenine or enhanced through the use of rapamycin, Chikungunya virus replication decreases or increases respectively, suggesting that autophagy supports Chikungunya virus replication (70).

While the earliest research papers on autophagy and viral infection often concluded that autophagy had been induced based on the observation that the number of autophagosomes or the amount of LC3-II had increased, more recent papers have distinguished between autophagosome accumulation due to autophagy induction, and due to blockage of lysosomal degradation of autophagosomes. For example,

autophagosome degradation is impaired in HCV infected cells, where autophagic degradation is not enhanced despite accumulation of autophagosomes (129), in poliovirus infected cells where autophagosomes are immobilised (139), and in influenza A virus infected cells where autophagosomes fail to fuse with lysosomes (37).

In some cases, viral determinants that modulate autophagy have been identified, for example poliovirus 2BC protein that when expressed alone, leads to covalent modification of LC3 (140), coronavirus non-structural protein 6 that activates autophagosome formation (20), and influenza A virus matrix protein 2 that blocks autophagosome-lysosome fusion (37). The poliovirus 2BC and 3A proteins are required together, not separately, in order for double-membrane vesicles which may be similar to autophagosomes, to be formed (134), and the modification of LC3 by 2BC presumably preceeds this. Since poliovirus 2BC and 3A are membrane-associated proteins, it could be that their overexpression induces ER stress, which may lead to autophagosome accumulation (55, 129).

That said, cellular mechanisms for autophagosome accumulation during viral infection remain largely unclear. Isolated papers report the dependence of autophagosome accumulation on PKR and eIF2α phosphorylation during herpes simplex virus type 1 (HSV-1) infection (136), and on the unfolded protein response / ER stress during Chikungunya virus and HCV infection (55, 129). However, the unfolded protein response / ER stress plays no role in the autophagosome accumulation caused by coronavirus infection as demonstrated by analysis of ER stress markers (20).

Autophagosomes may serve a proviral function in the case of the *Picornaviridae* family of viruses, since foot-and-mouth disease virus, coxsackievirus B3 and poliovirus all replicate more efficiently when autophagy is induced by rapamycin, and have reduced replication rates when autophagy is inhibited by 3-methyladenine (51, 94, 146). In foot-and-mouth disease virus and poliovirus infections, viral replication proteins also colocalise with autophagosome proteins, leading to the hypothesis that picornaviruses use autophagosome membranes as a scaffold for assembling replication complexes. Interestingly, coronaviruses appear to replicate on a special type of autophagy-independent, ER derived, LC3-I positive vesicle (112).

In other instances, autophagosomes may serve an antiviral function. Notably, virions or viral components may be targeted for autophagic degradation in a process termed xenophagy. HSV-1 virions are degraded by xenophagy (136). The ICP34.5 protein of HSV-1 antagonises xenophagy by binding Beclin 1 (95). The capsid protein of Sindbis virus has also been reported to be targeted for autophagic degradation through its interaction with p62/SQSTM1 (96), the cellular adaptor that links ubiquitinated protein aggregates to LC3. (However, in this case, it is not clear whether the targeting of Sindbis virus capsid protein was affected by any aggregation of capsid protein, since the capsid protein was linked to a bulky fluorescent marker and overexpressed before analysis of its co-localisation with LC3.)

Autophagy further contributes to the innate immune defence against viruses by helping plasmacytoid dendritic cells sense certain viruses. The sensor for viral single stranded

RNA in plasmacytoid dendritic cells is Toll-like receptor 7 (TLR7), located in endosomal/lysosomal compartments. UV-inactivated vesicular stomatitis virus (VSV), which is capable of being endocytosed but not capable of replication in the cytoplasm, does not activate TLR7. Live VSV is required for activation of TLR7, suggesting that replication intermediates in the cytoplasm are delivered to lysosomal compartments for sensing by TLR7. Autophagy provides this delivery system (74).

Autophagy also plays a role in the presentation of endogenous antigens on major histocompatibility complex (MHC) class II molecules. Classically, exogenous antigens taken up through endocytosis are loaded onto MHC class II molecules in late endosomal / MHC class II loading compartments. Autophagy delivers endogenous antigens to the MHC class II loading compartment, and targeting an endogenous antigen to autophagosomes enhances MHC class II presentation of that antigen (125). MHC class II presentation of endogenous Epstein-Barr virus nuclear antigen 1 depends on autophagy (98).

More recently, autophagy has also been implicated in MHC class I presentation of viral antigens. Autophagy has been suggested to enhance the presentation of HSV-1 antigens on MHC class I molecules in macrophages (29). In cells that lack transporter associated with antigen processing, which is necessary for the conventional MHC class I loading mechanism, autophagy was reported to enhance MHC class I presentation of human cytomegalovirus (HCMV) antigens (141). Interestingly, the autophagy process in melanoma cells has also been linked to enhanced cross-presentation (the presentation of exogenous antigens on MHC class I molecules) by dendritic cells, of antigens from the melanoma cells (77). Conceivably, autophagy-dependent MHC class I and class II presentation of virus-expressed antigens may contribute to CD8+ and CD4+ T cell immune surveillance, respectively.

1.3 MTOR

1.3.1 mTOR and rapamycin

The target of rapamycin, TOR was first discovered in *Saccharomyces cerevisiae* when mutations in TOR1 and TOR2 conferred resistance to the fungicide rapamycin, produced by the bacterium *Streptomyces hygroscopicus* (45). The naturally occurring rapamycin is used as an immunosuppressant that can regulate T cells and promote tolerance to grafts (152), and has also been found to increase longevity in mice (43).

The cellular receptor for rapamycin is FK506-binding protein 12 (FKBP12). The FKBPs are a family of immunophilins that bind immunosuppressive drugs such as FK506 and rapamycin (58). The mammalian counterpart of TOR, mTOR (also known as FKBP12-rapamycin-associated protein (FRAP), and rapamycin and FKBP12 target 1 (RAFT1)) was identified by its binding to rapamycin-FKBP12 and was found to be structurally and functionally conserved with its yeast counterparts (10, 115, 116).

mTOR is a large, 289 kDa serine/threonine kinase, which like the yeast TORs contains

a C-terminal catalytic domain with homology to phosphatidylinositol kinases, although no lipid kinase activity has been found for either yeast TORs or mTOR. mTOR also has a FKBP/rapamycin-binding domain, N-terminal to the catalytic domain, where rapamycin-FKBP12 binds (17). The binding of rapamycin-FKBP12 compromises mTOR kinase activity, reducing both its autophosphorylation (11) and its phosphorylation of substrates (13).

1.3.2 mTORC1 and mTORC2 are functionally distinct complexes

mTOR forms two distinct complexes, mTOR complex 1 (mTORC1) and mTORC2. mTORC1 comprises mTOR, raptor (regulatory-associated protein of TOR), mLST8 (also known as GßL), PRAS40 and DEPTOR (32, 62, 63, 97, 108, 120). mTORC2 comprises mTOR, rictor (rapamycin-insensitive companion of mTOR), mLST8, SIN1, protor and DEPTOR (49, 148). The raptor-containing mTORC1 is sensitive to rapamycin, whereas the rictor-containing mTORC2 is insensitive (50, 121).

mTORC1 integrates various signals such as nutrient availability, presence of growth hormones, oxidative stress, and energy status, and controls protein synthesis and cell growth, by phosphorylating substrates that are involved in the protein translation machinery (more below). Raptor serves as a scaffold for these substrates, binding to the TOR signalling (TOS) motif in certain substrates (93, 123, 124, 149).

The functions of mTORC2 are less well studied than those of mTORC1. mTORC2 controls the actin cytoskeleton and cell motility (121). It has also been reported to phosphorylate and activate Akt, an upstream positive regulator of mTORC1, meaning that mTORC2 could potentially regulate mTORC1 (122).

1.3.3 mTOR substrates S6K and 4E-BP1

The two most well characterised substrates of mTOR are p70 S6 kinase (S6K) and eIF4E binding protein 1 (4E-BP1) (11, 13). S6K and 4E-BP1 interact with raptor through their TOS motif, which is a conserved five amino acid segment, starting with Phe and alternating between an acidic and a hydrophobic residue. The TOS motif in the N-terminus of S6K is Phe-Asp-Ile-Asp-Leu, and that in the C-terminus of 4E-BP1 is Phe-Glu-Met-Asp-Ile (123).

Phosphorylation of S6K by mTOR activates it, allowing it to phosphorylate the 40S ribosomal subunit protein, S6, on five C-terminal serine residues. Phosphorylation of S6 is correlated with increased protein synthesis (52).

Hypo-phosphorylated 4E-BP1 is a negative regulator of translation intiation: it sequesters eIF4E, displacing it from its interaction with eIF4G and MAP kinase signal-integrating kinase 1 (Mnk1) in the eIF4F translation initiation complex. eIF4E recognises the 5’ cap structure of capped mRNA and eIF4G links mRNA to ribosomes (40). Mnk1 is a kinase that phosphorylates and activates eIF4E (145). Phosphorylation of 4E-BP1 at multiple sites, by mTOR, renders it inactive, freeing up eIF4E to bind

eIF4G and Mnk1, which allows for translation initiation. Phosphorylation of both S6K and 4E-BP1 by mTOR have the downstream effect of promoting cap-dependent translation (reviewed in (81)). The phosphorylation statuses of S6K/S6 and 4E-BP1 are commonly used as markers for mTORC1 activity.

1.3.4 Activation of mTORC1

mTORC1 responds to a number of different signals, including growth factors, energy status, hypoxia and nutrients. An important pathway for controlling mTORC1 activity is the class I PI3K-Akt-mTOR pathway (reviewed in (14)). Class I PI3K can be activated, for example, by insulin receptor substrates, in response to insulin (Figure 5). Class I PI3K then phosphorylates PI-4,5-bisphosphate, generating PI-3,4,5-triphosphate at the plasma membrane. Akt and its kinase phosphoinositide-dependent protein kinase 1 (PDK1) are recruited by PI-3,4,5-triphosphate to the plasma membrane, where PDK1 phosphorylates and activates Akt. Akt then inhibits the tuberous sclerosis complex (TSC), composed of TSC1 and TSC2. The TSC is a negative regulator of mTORC1, and is a GTPase activating protein that promotes the hydrolysis of Ras homolog enriched in brain (Rheb)-GTP to Rheb-GDP. Rheb-GTP activates mTORC1, by displacing the negative regulator of mTOR, FKBP38 (5, 24).

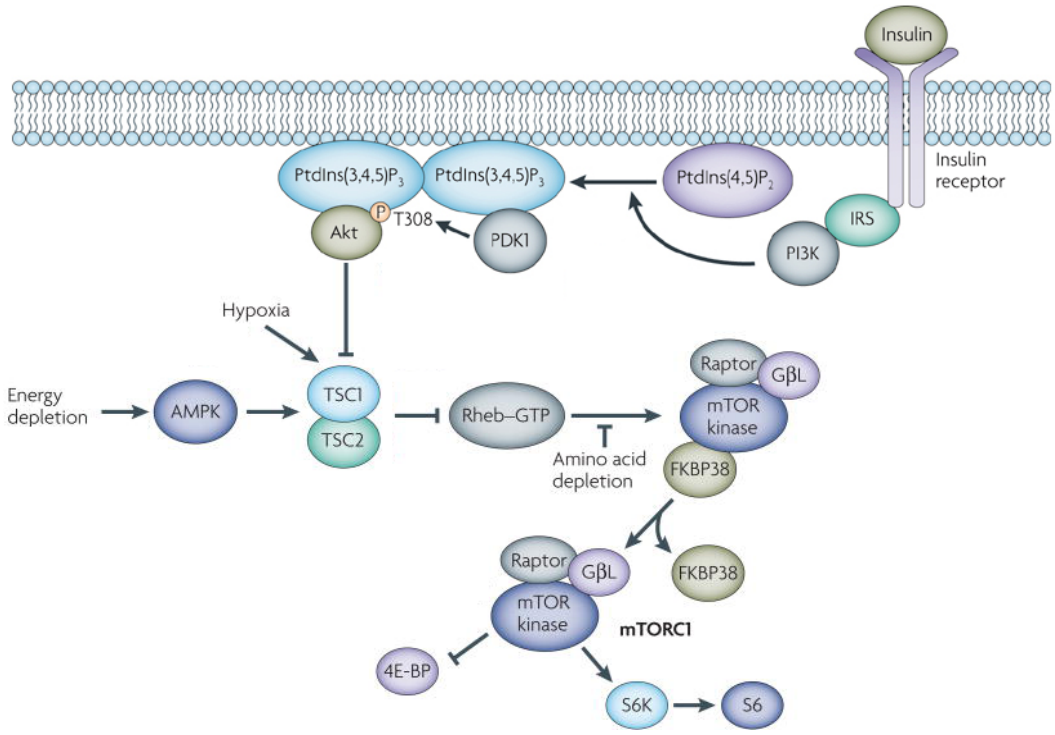

Figure 5

Schematic diagram showing the PI3K-Akt-mTOR pathway. Insulin binding to the insulin receptor leads to phosphorylation of insulin receptor substrates (IRS) that activate phosphatidylinositol 3-kinase (PI3K). PI3K phosphorylates PI-4,5-bisphosphate (PtdIns(4,5)P_2) to form PI-3,4,5-triphosphate (PtdIns(3,4,5)P_3) at the plasma membrane. Akt and phosphoinositide-dependent protein kinase 1 (PDK1) are recruited to the membrane by binding PtdIns(3,4,5)P_3. PDK1 phosphorylates Akt on threonine 308 (T308), activating Akt. Akt inactivates tuberous sclerosis complex 1 (TSC1)/TSC2, promoting hydrolysis of active Ras homolog enriched in brain (Rheb)-GTP to inactive Rheb-GDP. Rheb-GTP activates mTOR by displacing

FK506-binding protein 38 (FKBP38). Hypoxia, energy depletion and amino acid depletion regulate the pathway at the points indicated. Adapted from (14) with permission from Nature Publishing Group and J.C. Alwine.

The TSC is controlled not just by Akt, but also by AMP-activated kinase (AMPK) (66) (Figure 5). Under conditions of low energy in the cell, a high AMP to ATP ratio activates AMPK, which activates the TSC. Thus, mTORC1 is inactivated when cellular energy levels are low. mTORC1 is also inhibited under hypoxic conditions: hypoxia-inducible factor 1 promotes the transcription of protein regulated in development and DNA damage response 1, REDD1 which activates the TSC (12, 26).

The presence or absence of amino acids is the most ancient regulator of mTOR, and controls mTOR at a point in the signalling pathway most proximal to mTOR (Figure 5). The negative effect of amino acid depletion is dominant; insulin cannot activate mTORC1 if amino acids are absent (42). Amino acids promote the association of Rheb-GTP with mTOR (79).

The class III PI3K, Vps34, is a mediator of amino acid signalling to mTOR, although it is not known if Vps34 signals through Rheb or if it independently regulates mTORC1 (15, 21, 92). Another mediator of amino acid signalling is a family of four Ras-related GTP-binding (Rag) proteins, which form heterodimers - RagA and RagB can form heterodimers with RagC and RagD - and interact with mTORC1 (119). The Rag proteins enhance the interaction of mTORC1 and Rheb, by promoting mTORC1 translocation to lysosomal membranes that contain Rheb (118). While the expression of constitutively active mutants of Rag proteins causes a modest increase in S6K phosphorylation under nutrient rich conditions, the effect is much stronger under amino acid starved conditions, which suggests the involvement of Rag proteins in amino acid signalling (64). Consistent with the notion that Rag proteins are specifically involved in amino acid signalling, insulin-induced Akt phosphorylation is not affected by Rag proteins (64).

1.3.5 mTOR and viral infection

Viruses that cause acute infection often inactivate host cell cap-dependent translation and divert cellular protein synthesis to the synthesis of viral proteins. One way for these viruses to control cap-dependent translation is to modulate mTOR. For example, poliovirus mRNA has an internal ribosome entry site and thus does not depend on cap-dependent translation (104, 105). In poliovirus infected cells, eIF4G is cleaved and 4E-BP1 is dephosphorylated (41), the latter observation suggesting that mTOR is inactivated. In avian influenza A virus H5N1 infected cells, phospho-S6 levels are decreased in a TSC2-dependent manner (80), also suggesting that mTOR is inactivated. In cells infected with VSV, mTOR is inactivated via Akt dephosphorylation, even in the presence of growth factors or active PDK1. VSV matrix protein expressed alone leads to the dephosphorylation of Akt (25). Dephosphorylation of 4E-BP1 as a consequence of mTOR inactivation, in VSV infected cells, provides another pathway for inhibiting host cell translation apart from the phosphorylation of eIF2α (19). Interestingly, the inactivation of mTOR in VSV infected cells serves an antiviral

function that is dependent on the host stress response protein, growth arrest and DNA damage-inducible protein 34, GADD34 (86, 137).

Chronic viruses, on the other hand, may activate mTOR as part of a strategy to keep host cells alive and maintain viral persistence. For example, rapamycin-sensitive phosphorylation of S6K and 4E-BP1 is enhanced in Huh-7 human hepatoma cells harboring HCV subgenomic replicons. The HCV non-structural 5A protein is responsible for the activation of mTOR by competitively binding the mTOR inhibitor FKBP38, and the activation of mTOR mediates suppression of apoptosis (106). The replication of HCV is in turn restricted by mTOR activity, as S6K activates an antiviral protein, p21-activated kinase 1 (47). The restriction of HCV replication by activated mTOR limits viral replication to steady-state levels (82).

Another chronic virus, the DNA virus HCMV maintains mTOR activity in infected, amino acid starved cells, by causing Rheb and mTOR to localise to similar regions in the cell, in an amino acid-independent, Rag-independent, manner (18). HCMV also antagonises upstream negative regulation of mTOR by circumventing AMPK and TSC (71, 88).

An interesting comparison between acute and persistent infection is illustrated by differential effects on mTOR activation status in Sindbis virus infected mammalian versus insect cells. Sindbis virus causes cytopathic and acute infection in mammalian cells but a less cytopathic and more persistent infection in insect cells. 4E-BP1 phosphorylation, and cap-dependent translation, is increased in Sindbis virus infected mosquito, but not mammalian, cells (103). The activation of mTOR in infected insect but not mammalian cells may play a role in establishing Sindbis virus persistence in insect cells.

Components of the immune response to viral infections may interact with the mTOR pathway. Type I IFN signalling, for example, has been shown to lead to downstream phosphorylation of S6K and 4E-BP1, in a manner that is dependent on the functions of PI3K and mTOR (75). Further, mTOR activity may play a role in supporting the translation of IFN-stimulated genes. For instance the induction of a key IFN-inducible protein, IFN-stimulated gene 15, is enhanced in 4E-BP1, TSC1 or TSC2 knockout cells (59). Consistent with the notion that mTOR activity may support IFN responses, knocking out Akt results in a dramatic reduction in IFN-induced antiviral responses (60).

2 AIMS OF THESIS

2.1 GENERAL AIMS

The general aim of this thesis was to better understand the roles of autophagy (Paper II) and mTOR (Paper III) during SFV infection. To be better equipped to study autophagy, we also aimed to develop a method for quantifying autophagosomes that would be more efficient than available methods at the time (Paper I).

2.2 SPECIFIC AIMS

Since autophagy promoted picornavirus replication (51, 94, 146), in Paper II we specifically aimed to determine whether autophagy also promoted SFV replication. Also, since Sindbis virus capsid protein had been reported to be targeted for xenophagy (96), we aimed to investigate whether any SFV proteins were targeted to autophagosomes. Further, we aimed to define the viral determinant responsible for the autophagosome accumulation that we observed in SFV infected cells. Since autophagy is a dynamic process involving constant synthesis and degradation of autophagosomes, we also aimed to decipher whether SFV-dependent autophagosome accumulation was the result of increased autophagy induction, or decreased autophagosome degradation.

In Paper III, we aimed to define the differences in mTOR activation status in SFV infected versus non-infected cells, under nutrient rich and amino acid starved conditions. We also aimed to understand the effect of mTOR activity on SFV replication rate, and to pinpoint which viral components may be necessary for the maintenance of mTOR activity in amino acid starved cells that we observed. Further, we aimed to investigate the role of various components of the PI3K-Akt-mTOR pathway in SFV-dependent maintenance of mTOR activity.

3 RESULTS AND DISCUSSION

3.1 PAPER I

In order to better study autophagy, we developed a novel flow cytometry-based method for the quantification of autophagosomes in Paper I (27). We generated a HOS cell line that stably expressed the EGFP-tagged autophagosome marker, EGFP-LC3. HOS-EGFP-LC3 cells that were rinsed with low concentrations of saponin (0.05% w/v) became permeabilised in such a way that non-membrane-bound, cytoplasmic EGFP-LC3-I, but not autophagosome-associated EGFP-LC3-II, was lost in the saponin wash (Figure 6A). The saponin rinse made it possible to specifically detect EGFP-LC3-II, rather than total EGFP-LC3, by flow cytometry. Since EGFP-LC3-II and not total EGFP-LC3 is the actual marker for autophagosomes, our novel method provided a means for indirectly quantifying autophagosomes through the quantification of EGFP-LC3-II, by flow cytometry. We confirmed that the saponin rinse specifically caused the loss of EGFP-LC3-I, by immunofluorescence and western blot analyses.

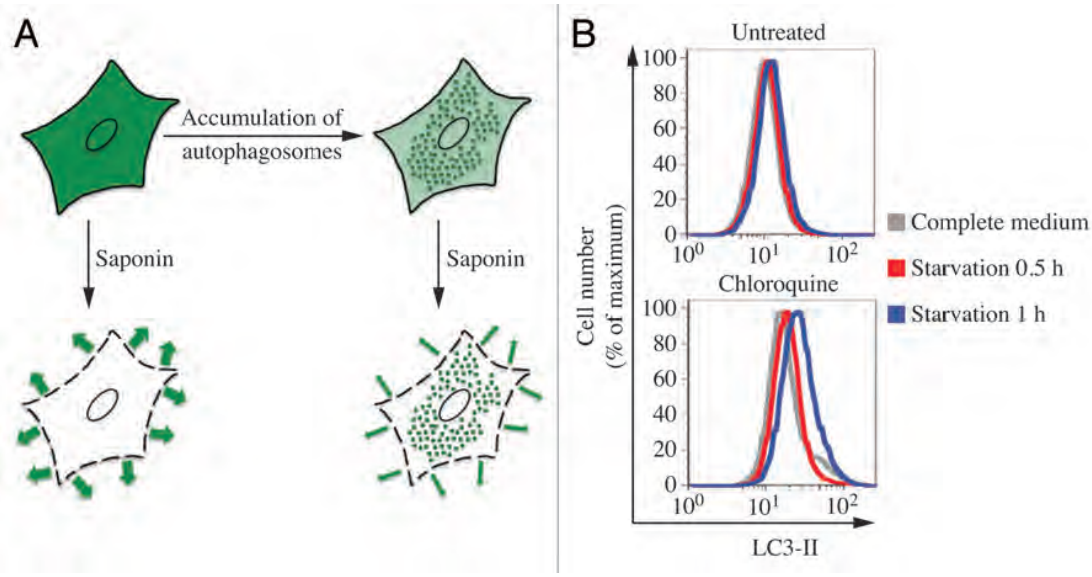

Figure 6

Saponin rinse and quantification of LC3-II by flow cytometry. (A) Schematic diagram showing the effects of a saponin rinse. Accumulation of autophagosomes may entail a redistribution of enhanced green fluorescent protein (EGFP)-LC3, as cytoplasmic EGFP-LC3-I is converted to autophagosome-associated EGFP-LC3-II (top). This does not cause changes in total levels of EGFP-LC3 (i.e. EGFP-LC3-I + EGFP-LC3-II remains constant), and flow cytometry analysis will not differentiate between the EGFP fluorescence intensities of the two cells in the top half of the diagram. With a saponin rinse, cytoplasmic EGFP-LC3-I is extracted, while autophagosome-bound EGFP-LC3-II remains in the cell. This allows for differentiation between the two cells: a greater fluorescence signal will be detected in the cell on the bottom right than on the bottom left. (B) Flow cytometry histograms showing starvation-induced endogenous LC3-II accumulation. Human osteosarcoma (HOS) cells were mock treated or treated with 50 µM chloroquine for 1 hour while simultaneously incubated in complete medium or

starved of amino acids and serum by incubation in Earle's balanced salt solution (EBSS) for the last 0.5 or 1 hour. Cells were then rinsed with saponin and stained for LC3. Previously published in (68).

We amino acid starved, or chloroquine treated, HOS-EGFP-LC3 cells to influence the number of autophagosomes in the cell. Chloroquine is a small molecule that translocates into the lysosome and sequesters protons, neutralising the acidity that is required for lysosomal proteases to function, thus blocking autophagosome degradation and increasing the number of autophagosomes. We measured autophagosomes with our flow cytometry-based method as well as with pre-existing methods based on immunofluorescence or western blot analysis, and obtained comparable results across the different methods. Our flow cytometry-based method had the advantage of being less subjective, less time consuming and less labour intensive than the pre-existing methods.

We also used our method to measure new synthesis of autophagosomes over time, by using ammonium chloride, NH_4Cl to inhibit autophagosome degradation. NH_4Cl, like chloroquine, blocks autophagosome degradation by neutralising lysosome acidity. We showed that adding NH_4Cl to starved or rapamycin-treated cells allowed us to easily determine the levels of starvation-induced, or rapamycin-induced autophagy, with our flow cyometry-based method.

Further, we extended our method to the detection of endogenous LC3 by incubating saponin-rinsed HOS cells as well as saponin-rinsed mouse embryonic fibroblasts (MEFs) with an LC3-specific antibody followed by a fluorescent marker conjugated secondary antibody. We were able to detect endogenous LC3-II in HOS cells, and in wild type $atg5^{+/+}$ MEFs, but not in $atg5^{-/-}$ MEFs.

Combining the above two applications of our new method, we were able to detect starvation-induced, endogenous LC3-II, by starving HOS cells while treating them with chloroquine to prevent degradation of autophagosomes, and then rinsing the cells in saponin before staining for LC3 (Figure 6B).

Taken together, these results indicated that our novel method was useful for quick and non-subjective quantification of autophagosomes and analysis of autophagy induction, and that it had potential to be used in any cell type, transfected or non-transfected.

We also observed that amino acid starvation caused little increase in EGFP-LC3-II levels in HOS cells unless NH_4Cl was present, leading us to believe that the degradation rate of autophagosomes increased concurrently with autophagy induction rates, during amino acid starvation, in HOS cells. We speculated that HOS cells had an intrinsically high capacity for degradation of autophagosomes. This was instructive for development of ideas in Paper II about the nature of SFV-induced autophagosome accumulation (see next section).

3.2 PAPER II

In Paper II, we studied autophagy in the context of SFV infection (28). We observed

that autophagosomes increased up to 12 hours post infection (hpi) in SFV infected cells (Figure 7). This observation was aided by the method developed in Paper I (Figure 7C).

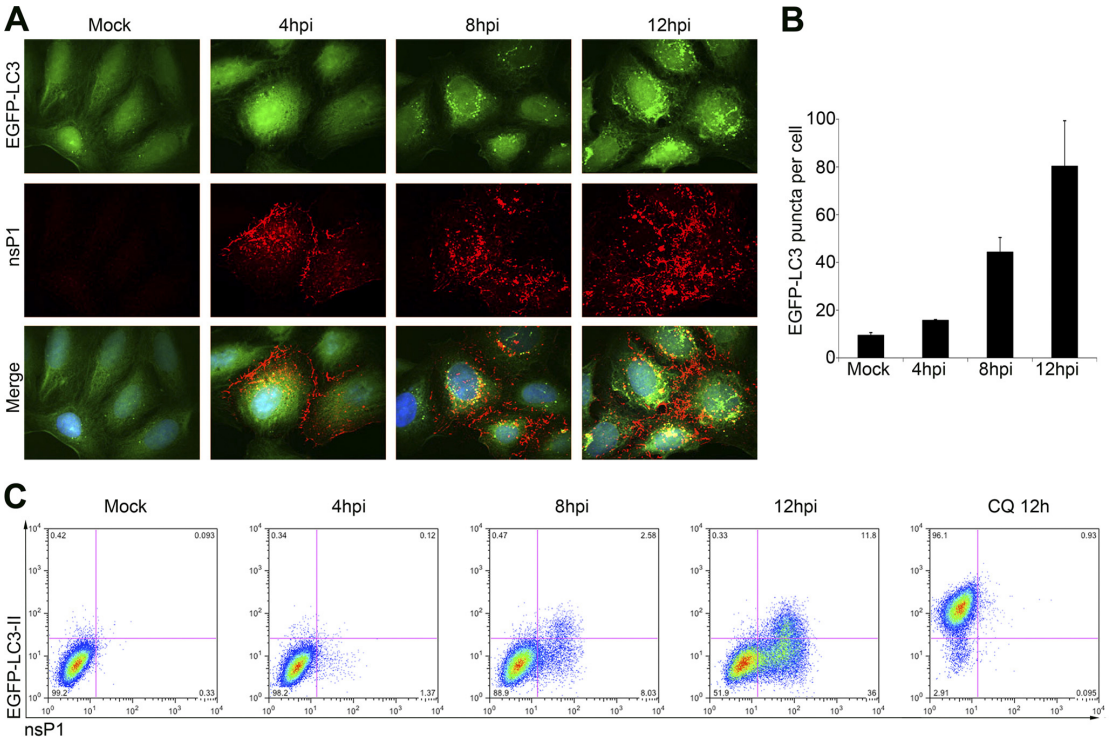

Figure 7

Autophagosomes accumulate in SFV infected cells. (A) HOS-EGFP-LC3 cells were infected with SFV. EGFP-LC3 positive puncta increased over time, in infected cells staining positive for SFV nsP1. (B) Quantification of data presented in (A). (C) Flow cytometry analysis of cells infected as in (A) or treated, as a positive control, with 50 μM chloroquine, showing EGFP-LC3-II accumulation in nsP1 positive cells but not nsP1 negative cells within the same SFV-treated sample. Previously published in (28).

Questioning the role of these accumulated autophagosomes, we examined SFV replication rates in autophagy competent ($atg5^{+/+}$) and incompetent ($atg5^{-/-}$) MEFs. There was no difference in SFV replication rates between the two cell lines, suggesting that unlike the picornaviruses (51, 146), SFV did not derive a replicative advantage from the increased level of autophagosomes. We note that Chikungunya virus replication has also been reported to be enhanced by the presence of autophagosomes (albeit to a modest extent) (70), and we did not observe the same effect for SFV viral replication even though Chikungunya virus and SFV are closely related. The replication rate of another closely related virus, Sindbis virus is not affected by the presence of autophagosomes (96).

We further examined whether SFV proteins were targeted to autophagosomes, since 1. poliovirus assembled its replication complexes on autophagosomes (51) and 2. Sindbis virus capsid protein was targeted for autophagic degradation via interaction with p62/SQSTM1 (96). We found that the nsPs and structural proteins of SFV colocalised either only sparingly with autophagosome markers or not at all, suggesting that SFV neither utilised autophagosome membranes for replication to any meaningful extent, nor had its components targeted for autophagic degradation. These conclusions were consistent with the earlier observation that viral replication rates were not affected by

the presence of autophagosomes.

To define whether autophagosome accumulation during SFV infection was caused by enhanced autophagosome formation or a blockage in autophagosome degradation, we employed two methods. First, we assessed the activation status of mTOR, since mTOR is a well-known repressor of autophagy. Amino acid starvation induces autophagy by lifting the repression caused by mTOR, as discussed in section 1.2.1. We found that mTOR remained active in SFV infected cells, suggesting that at least autophagy was not enhanced by inactivation of mTOR during SFV infection. It remained possible at this point that autophagy was enhanced by other mechanisms during infection.

Second, we assessed to what extent new autophagosomes were synthesised during SFV infection, by blocking degradation of autophagosomes with addition of NH_4Cl, a protocol we had established in Paper I. We found that while amino acid starved cells accumulated a greater number of autophagosomes in the presence of NH_4Cl than in its absence, representing starvation-induced, newly synthesised autophagosomes, SFV infected cells did not accumulate more autophagosomes in the presence of NH_4Cl compared to in its absence. This suggested that the increase in autophagosomes in SFV infected cells was due to a blockage in autophagosome degradation, rather than enhanced formation of new autophagosomes.

Since we had observed in Paper I that the degradation capacity for autophagosomes was high in HOS cells and an increase in the formation rate of autophagosomes (due to starvation or other factors) was not likely to lead to observable increases in LC3-II levels without addition of autophagosome degradation inhibitors, it was not surprising that SFV infection, which alone caused a substantial observable increase in LC3-II, caused autophagosome accumulation by blocking autophagosome degradation.

To confirm that autophagosome degradation was impaired in SFV infected cells, we made use of the tandem reporter mRFP-EGFP-LC3 (67). Since EGFP is more sensitive to acidic conditions than mRFP, the mRFP-EGFP-LC3 loses its EGFP, but not mRFP, signal when it has been delivered to acidic lysosomes. The ratio of yellow (autophagosomes not yet fused with lysosomes) to red (autophagosomes fused with lysosomes where acidic pH bleached the EGFP signal) LC3 puncta was greatly increased in SFV infected cells compared to in mock infected cells, providing further evidence that autophagosome degradation was blocked in SFV infected cells.

Gannagé et al. reported that the influenza A virus matrix protein 2 (M2) blocks autophagosome degradation (37). M2 is a proton channel, leading the authors to hypothesise that it was able to block acidification of lysosomes. However, inhibiting the proton channel function of M2 did not prevent it from causing autophagosome accumulation, suggesting that another function of M2 was involved. The SFV 6K protein has ion channel activity (84), making it possible that 6K might block autophagosome degradation by preventing lyosomal acidification.

To define which viral components were necessary for autophagosome accumulation, we infected HOS-EGFP-LC3 cells with various recombinant SFV (rSFV) variants (Figure 8). Infection with SFVΔ6K, which lacked the region encoding both 6K and

Transframe, caused similar autophagosome accumulaton as infection with wild type SFV, suggesting that neither 6K nor Transframe was the protein responsible for autophagosome accumulation in SFV infected cells. We observed instead that the rSFV variants that did not express spike proteins (SFVΔspike and SFV-ßgal), did not cause accumulation of autophagosomes (Figure 8). Thus we concluded that the expression of SFV spike proteins was necessary for the accumulation of autophagosomes observed in SFV infected cells. Viral RNA replication was not the causative agent of autophagosome accumulation, as SFV-ßgal, which has the genes for SFV structural proteins replaced with the gene encoding *Escherichia coli* ß-galactosidase, expressed the nsPs necessary for viral RNA replication but did not cause an accumulation of autophagosomes in infected cells.

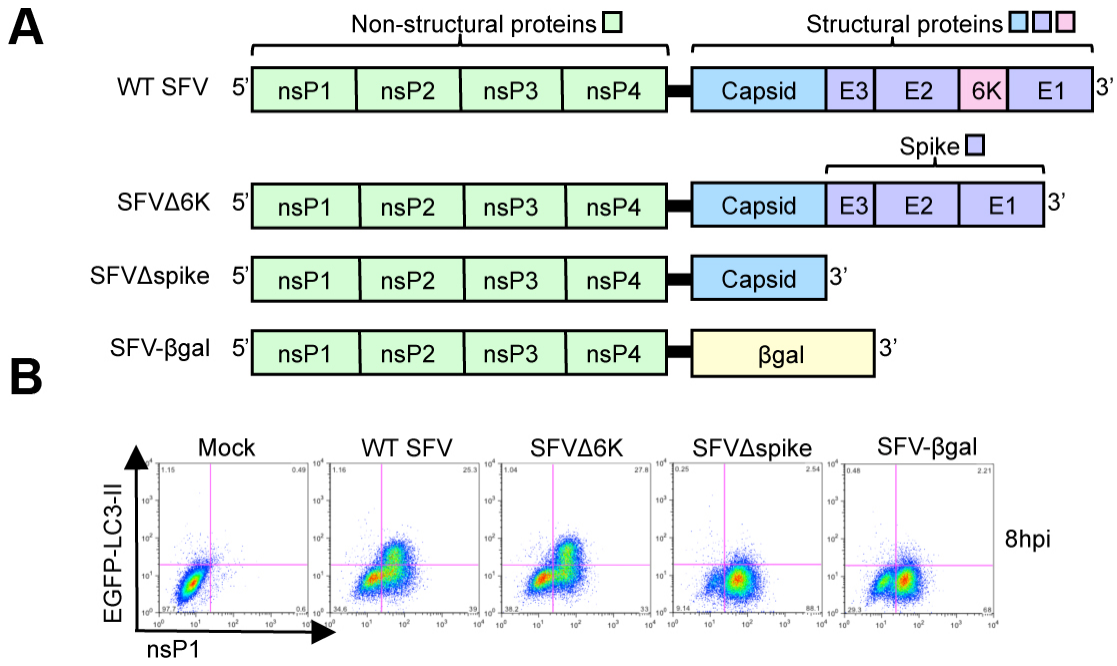

Figure 8

Autophagosome accumulation depends on expression of SFV spike proteins. (A) Schematic diagram showing the genes of recombinant SFV (rSFV) variants. SFV-ßgal lacks all SFV structural genes; in place of the structural genes is the gene encoding *Escherichia coli* ß-galactosidase. (B) Flow cytometry analysis of HOS-EGFP-LC3 cells infected with the rSFV variants shown in (A). Only infection with rSFV variants expressing SFV spike proteins caused EGFP-LC3-II accumulation. Part (B) was previously published in (28).

A further experiment that could confirm the role of SFV spike protein expression would be to test if a plasmid encoding SFV spike proteins, and no other SFV proteins, would cause autophagosome accumulation when transfected into cells. Since we have not performed this experiment, we cannot claim that expression of SFV spike proteins is sufficient for autophagosome accumulaton, just that it is necessary. Thus, we cannot exclude the possibility that the effect of expression of SFV spike proteins on autophagosome accumulation can only be observed in an infected cell, due to cooperation by some other viral or cellular factor.

A mechanism by which expression of SFV spike proteins leads to autophagosome accumulation is yet to be elucidated. SFV spike proteins are synthesised in the ER at high levels causing ER stress (7). Since ER stress has been reported to mediate autophagy induction during HCV infection and Chikungunya virus infection (55, 129), it is possible that the expression of SFV spike proteins causes autophagosome accumulation via ER stress. To address this, in unpublished work we have infected HOS-EGFP-LC3 cells with wild type SFV or SFV-ßgal, or treated HOS-EGFP-LC3 cells with thapsigargin. We found that the level of ER stress caused by the ER stressor thapsigargin far exceeded that caused by wild type SFV infection, as measured by the ER stress marker, spliced X-box-binding protein 1 mRNA (Figure 9). However, the amount of EGFP-LC3-II accumulation caused by thapsigargin treatment was far less than that caused by infection with wild type SFV. SFV-ßgal infection did not induce ER stress, as expected since spike proteins are not expressed by SFV-ßgal. The levels of autophagosome accumulation caused by thapsigargin treatment and caused by infection with the non ER stress inducing SFV-ßgal were comparable. Since there was a lack of correlation between ER stress levels and autophagosome accumulation in this experiment, we believe that even if ER stress contributes partly to SFV-dependent autophagosome accumulation, there must be other mechanisms involved that play a more important role.

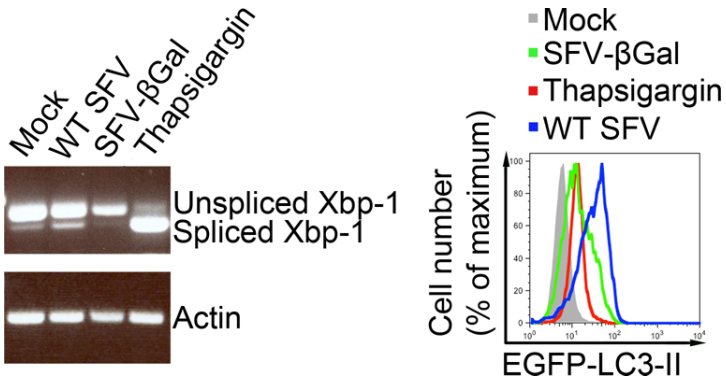

Figure 9

Comparison between SFV infection and the ER stressor thapsigargin. Left, DNA gel electrophoresis of cDNA reverse transcribed from X-box-binding protein 1 (Xbp-1) mRNA, from HOS-EGFP-LC3 cells infected with wild type SFV or SFV-ßgal, or treated with thapsigargin at 5 µM, for 6 hours. Right, histograms from flow cytometry analyses of HOS-EGFP-LC3 cells infected with wild type SFV or SFV-ßgal for 14 hours, or treated with thapsigargin at 5 µM for 10 hours.

3.3 PAPER III

In non-published work done for Paper II, we hypothesised that if amino acid starvation induced autophagy and SFV infection blocked the degradation of autophagosomes, a larger number of autophagosomes would accumulate in cells that were both SFV infected and amino acid starved, than in cells that were only SFV infected. To our consternation, we did not observe the expected result. The amount of EGFP-LC3-II measured in SFV infected, amino acid starved cells was no greater than that measured in cells that were only SFV infected (Figure 10).

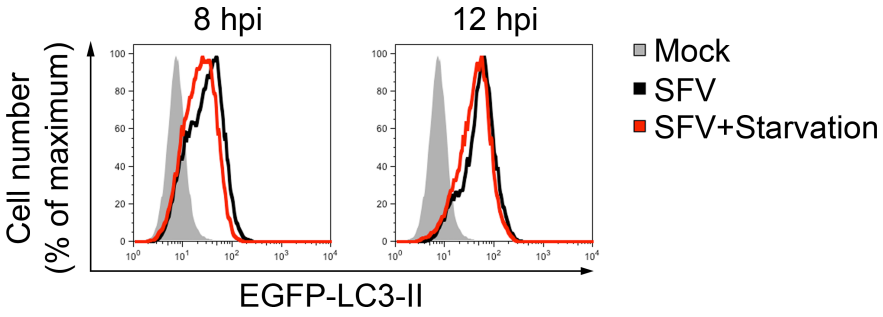

Figure 10

Flow cytometry histograms showing the (lack of) effect of starvation on autophagosome accumulation in SFV infected cells. HOS-EGFP-LC3 cells were infected with SFV and starved by incubation in EBSS at 1 hour post infection (hpi) or incubated in normal medium. At the indicated times, cells were harvested, saponin rinsed and analysed for EGFP-LC3-II by flow cytometry. SFV infected cells, both starved and not starved, were gated based on nsP1 positive staining.

This result led us to question whether amino acid starvation induced autophagy in SFV infected cells as it does in non-infected cells. Starvation induces autophagy by inactivating mTOR. To test the effect of amino acid starvation on mTOR activity status in SFV infected cells, we mock infected or SFV infected HOS cells and at 4 hpi changed the medium to normal medium, or to Earle's balanced salt solution (EBSS) to starve the cells of amino acids and serum. We lysed the cells after a further 4 hours, at 8 hpi, and analysed the lysates. We found that although amino acid starvation led to dephosphorylation of mTOR, S6, and 4E-BP1 in mock infected cells as expected, it did not do so in SFV infected cells (Figure 11). The maintenance of mTOR activity in SFV infected, starved cells, explained why we did not observe starvation-induced autophagy in these cells.

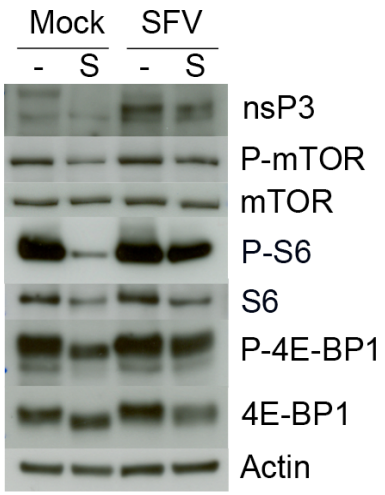

Figure 11

Western blot analysis showing maintenance of mTOR activity in SFV infected cells during amino acid starvation. HOS cells were mock infected or infected with SFV. At 4 hpi, cells were incubated in normal medium (-), or amino acid starved (S) by incubation in EBSS. After a further 4 hours, lysates were

obtained and analysed by western blot for the indicated proteins. Reproduced from Paper III (manuscript).

We wondered whether the maintenance of mTOR activity in SFV infected cells played a role in viral growth. Since we observed that mTOR remained sensitive to rapamycin in SFV infected cells as evidenced by rapamycin-induced dephosphorylation of S6 in infected cells, we made use of rapamycin to test the effect of mTOR activity on SFV growth. We infected HOS cells with SFV, then mock treated or added rapamycin to the cells at 4 hpi, and measured the amount of progeny virus at intervals over a 48-hour time course. We found that SFV growth rate was not affected by rapamycin under these conditions.

We speculated that an SFV infected cell might face a situation approaching amino acid starvation during the course of infection, due to the high amounts of viral proteins synthesised, compounded by the inhibition of autophagic degradation (Paper II), which would normally help to recycle amino acids in non-infected cells. Thus, suspecting that mTOR activity might confer a replicative advantage to SFV specifically in the event of amino acid starvation, we infected HOS cells with SFV and mock treated or added rapamycin to the cells, while simultaneously changing the medium to normal medium, EBSS, or mixtures of both across a gradient of normal medium: EBSS ratios. We found that cells incubated in EBSS produced far less progeny virus than cells incubated in normal medium or in a mixture of EBSS and normal medium. Rapamycin, however, had no effect on viral growth, whether the cells were incubated in EBSS, normal medium or a mixture of both. These results suggested that while the presence of amino acids was crucially important for SFV growth, mTOR activity was expendable. It is possible that the maintenance of mTOR activity might be a host defence response, for example by supporting the expression of IFN-stimulated genes (59, 60). Many mTOR-dependent antiviral effects have been proposed (1, 3, 47, 86, 127, 137).

The phosphorylation of eIF2α in SFV infected cells compromises mRNA translation but the SFV 26S subgenomic mRNA has an enhancer sequence that evades this effect (83). Since we found that the dephosphorylation of S6 and 4E-BP1 caused by rapamycin treatment had no effect on SFV growth, it seems possible that SFV subgenomic mRNA also circumvents low ribosomal activity and/or low concentrations of active eIF4F complex. It has been reported that translation of VSV mRNAs is not affected by low concentrations of active eIF4F complex when 4E-BP1 is dephosphorylated in VSV infected cells (19), an intriguing finding for a virus with 5’ capped mRNA, since eIF4E is the cap-recognising component of the cellular translation machinery.

Viral replication was found to be necessary for the mTOR-activating effect observed in Sindbis virus infected insect cells, as infection with UV-inactivated Sindbis virus failed to increase phosphorylation of 4E-BP1 in these cells (103). To test whether SFV replication was necessary for mTOR activity maintenance in our system, we compared the effect of UV-inactivated SFV and non-UV-inactivated SFV. Incubation of HOS cells with UV-inactivated SFV, unlike with SFV, did not lead to maintenance of mTOR activity during amino acid starvation, suggesting that viral replication was required for the effect observed in SFV infected cells.

To define the viral determinants that caused maintenance of mTOR activity, we infected HOS cells with wild type SFV or SFV-ßgal. As described previously, SFV-ßgal expresses the viral nsPs and undergoes RNA replication, but it does not express any viral structural proteins. The effect on mTOR activation status was similar between infection with wild type SFV and infection with SFV-ßgal, suggesting that expression of SFV structural proteins was not necessary for maintenance of mTOR activity. In Paper II we had showed that expression of SFV spike proteins was necessary for autophagosome accumulation, and we note here that the effect of SFV infection on mTOR activity is unrelated to the spike-dependent effect on autophagosomes.

As the class I PI3K-Akt-mTOR signalling pathway plays a significant role in signal transduction to mTOR, we wondered if class I PI3K was involved in SFV-dependent maintenance of mTOR activity. We found that addition of wortmannin, a PI3K inhibitor, abrogated the effect of SFV on the maintenance of mTOR activity in amino acid starved cells. Wortmannin alone did not cause mTOR to be inactivated. This result suggested that a wortmannin sensitive, PI3K-dependent mechanism was involved in signal transduction to mTOR in SFV infected cells.

Interestingly, we found that whereas wortmannin did not, LY294002, another PI3K inhibitor, inactivated mTOR when added alone. However, LY294002 has other targets than PI3Ks (39), and it is possible that the effect of LY294002 on mTOR inactivation occurs via these other targets.

Since wortmannin can inhibit both class I and class III PI3K (99, 109), it is also possible that the wortmannin sensitivity of the SFV effect is not due to involvement of class I PI3K (via the class I PI3K-Akt-mTOR pathway), but due to involvement of class III PI3K. It is known that the mammalian class III PI3K is highly sensitive to wortmannin (109), and in particular more sensitive than its yeast counterpart (143), although it is not known if mammalian class III PI3K is more sensitive to wortmannin than class I PI3K. A potential mediator of the SFV-dependent effect could thus be the class III PI3K Vps34, which is normally involved in amino acid dependent activation of mTOR (15, 92).

SFV causes acute cytopathic infection in mammalian cell lines, with extensive cell death and high output of progeny virus. We were surprised when SFV infection of mammalian cells led to maintenance of mTOR activity despite amino acid starvation, much like the phenotype observed with the chronic DNA virus HCMV (18). Sindbis virus, which is closely related to SFV, did not enhance mTOR activity above constitutive levels in infected mammalian cells, but it is not known whether mTOR activity would be maintained in these cells under conditions of amino acid starvation (103). To our knowledge, the work in paper III is the first documentation of an acute RNA virus infection that has positive effects on mTOR activity. Further studies would be required to shed light on the biological significance of this modulation. Also, it would be interesting to find out whether SFV infection renders mTOR resistant to the effects of other negative regulators such as energy depletion and hypoxia.

4 CONCLUSION

The development of a novel technique for measuring autophagosomes in Paper I provided a useful and reliable tool for studying autophagy. This technique was utilised to a great extent in Paper II. We also contributed a figure to the most recent "Guidelines for the use and interpretation of assays for monitoring autophagy" based on this technique (68). While not necessarily more sensitive than pre-existing methods, our flow cytometry-based assay is quicker and less subjective.

Both autophagy and (m)TOR activity are fundamental and ancient biological processes conserved in all eukaryotic cells. The work done in Paper II and Paper III of this thesis describes aspects of these fundamental processes in SFV infected cells. Briefly, our findings can be summarised as follows:

1. Autophagosomes accumulate in SFV infected cells
2. The degradation of autophagosomes is impaired in SFV infected cells
3. Expression of SFV spike proteins is necessary for the accumulation of autophagosomes
4. mTOR activity is maintained in SFV infected, amino acid starved cells
5. mTOR activity maintenance does not depend on expression of SFV structural proteins
6. Inhibition of mTOR activity by rapamycin has no effect on SFV growth

How the expression of SFV spike proteins mediates autophagosome accumulation, whether mTOR activity maintenance in SFV infected, amino acid starved cells is a result of a host defence mechanism, and how mTOR activity is maintained in infected, starved cells, remain to be elucidated.

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